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FLUORESCENCE MICROSCOPY FOR ALGAL STUDIES

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## FLUORESCENCE MICROSCOPY FOR ALGAL STUDIES

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### ABSTRACT

Fluorescence-inducing illumination in combination with conventional tungsten white light provided enhanced microscopical observations of algae. Maximum fluorescence emission of bili-protein-containing algae (Cyanophyta, Rhodophyta, and Cryptophyta) occurred with green excitation light ( $\sim 540$  nm). Bacillariophyta, Chlorophyta, Chrysophyta, and Euglenophyta fluoresced brightest when excited with blue-violet light (380-490 nm).

A comparison of preservation techniques showed that gluteraldehyde was superior to treatment with either formalin, "M<sup>3</sup>", or freezing samples for examination by fluorescence microscopy.

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## INTRODUCTION

Fluorescence microscopy is frequently used in aquatic bacteriological studies, but an extensive literature search revealed few published accounts of its use in routine algal studies. All algae and higher plants contain chlorophyll *a* and show primary fluorescence in the visible spectrum. Photosynthetic bacteria, which contain bacteriochlorophyll, fluoresce in the infrared portion of the spectrum (Pierson and Howard, 1972) unless they are treated with special staining techniques (Fliermans and Schmidt, 1975).

The applicability of fluorescence microscopy to routine algal studies was initially discussed by Wood (1962). He stated that it was possible to easily distinguish between photosynthetic (chlorophyll-bearing) flagellates and the occasionally more-numerous, non-pigmented flagellates. Brock (1968) used fluorescence microscopy to study thermophilic bacteria and blue-green algae. These studies indicated that several small-diameter filamentous organisms previously classified as blue-green algae were actually bacteria. More recently, Jones (1974) demonstrated that epifluorescence microscopy provides reliable estimates of epilithic diatom quantities, and Brock (1978) described a technique for examining primuline-stained phytoplankton with a fluorescence microscope following membrane filtration.

The ability of chlorophyll molecules to emit fluorescent light has been exploited for several years in algal studies

involving fluorometric chlorophyll estimates of standing crop (Yentsch and Menzel, 1963; Holm-Hansen, *et al.*, 1965; Kiefer, 1973), but fluorescence has been only sparingly used in studies involving microscopic examinations. This paper describes how epifluorescence microscopy facilitates examinations of algae and discusses advantages the technique provides over the conventional use of white-light illumination alone.

## MATERIALS AND METHODS

### Mechanics of Incident-Light Fluorescence

Major advances in fluorescence microscopy used in this work included (1) the recent development of epifluorescence microscopy to replace transmitted fluorescence and (2) the development of high-quality interference filters to replace less-specific glass filters.

Figure 1 illustrates the basic pattern of epifluorescence light passage using a filter set which resulted in good chlorophyll fluorescence with blue-light excitation. A BG-38 (heat filter) suppresses most of the UV light emitted by the mercury light source. Light below 455 nm and above 490 nm is restricted by exciter filters LP-455 and KP-490, respectively. The remaining band of blue light is reflected off a chromatic beam splitter (FL-510) and passed through the objective lens (which serves as a condenser) to the specimen. Bright red chlorophyll fluorescence resulting from the blue excitation of the chlorophyll-containing specimen passes through the objective lens, chromatic beam

splitter, and barrier filter (LP-520) to the ocular lenses. The barrier filter restricts light below 520 nm, thus eliminating background illumination and overlap of exciting and fluorescence radiation. Fluorescing specimens were often simultaneously illuminated by transmitted conventional white light.

### Basic Equipment

The basic microscope used in this study was a Zeiss Invertoscope D-FL containing an IV FL Epifluorescence condenser. Fluorescence-inducing illumination was provided by an HBO 50W super-pressure mercury vapor lamp, while white light was provided by a 15-watt tungsten filament lamp. The epifluorescence condenser contained a sliding chamber with two stop positions for two reflector sleeves, each containing a complete filter set. The ability to instantly exchange two complete filter sets (exciters, chromatic beam splitters, and barriers) made it possible to observe the chlorophyll fluorescence of a selected cell exposed to two different excitation wave lengths before the fluorescence began to fade.

### Filters and Objective Lenses

Maximum chlorophyll fluorescence reportedly occurs at excitation wave lengths between 430 and 460 nm (Yentsch and Menzel, 1963; Tunzi, *et al.*, 1974). However, the standard Zeiss filter sets designed for the fluorochromes FITC and Rhodamine with excitation wave lengths of 380 to 490 nm and 520 to 560 nm,

respectively, were found to be quite applicable for algal examinations. Various combinations of filters were tested for their effect on chlorophyll fluorescence emission using algal samples collected from a variety of lotic and lentic habitats. Oil immersion 40X or 63X planapochromatic objective lenses provided brighter fluorescence observations than did neofluar lenses of the same magnifications and were thus used for most observations in this study.

### Preservation Techniques

Because it is seldom feasible to examine live cells in quantitative algal surveys, a variety of preservation techniques were evaluated for use with fluorescence microscopy. Algae for this experiment were obtained by subdividing a thoroughly-mixed 15-L phytoplankton sample into 250-ml subsamples. The subsamples were randomly selected for a variety of preservation procedures which included the addition of 3% sodium tetraborate-buffered formalin, 5% buffered formalin, 5% "M<sup>3</sup>" solution (Meyer, 1971) and 5% gluteraldehyde solution (50% w/w). Some subsamples were frozen without a chemical preservative. The initial homogeneity among subsamples was assessed within a few days of collection. Using the inverted microscope methods of Lund, *et al.*, (1958), algal populations in three subsamples and from four 10-ml aliquots of the same subsample yielded no significant differences.

The subsamples used for the preservation technique comparisons were placed in a refrigerator within a few hours of

collection and were stored at about 5°C; subsamples without a chemical preservative were stored at -18°C. All subsamples were stored for approximately nine months. After the nine-month storage, the subsamples were then examined with and without the use of fluorescence illumination. Inverted microscope enumerations were made and all algae were categorized as "live" or "dead" (Webber, 1973). Cells were considered "live" if they exhibited chlorophyll fluorescence or if they contained visible pigmentation when only white light illumination was used.

## RESULTS

### Intensity of Fluorescence at Different Excitation Wavelengths

The brightest fluorescence of blue-green algae (Cyanophyta), red algae (Rhodophyta), and cryptomonads (Cryptophyta) observed in this study always occurred with the green excitation wavelengths (520 to 560 nm) of the Rhodamine filter set, whereas, all of the other algae examined fluoresced much brighter when excited with the blue-violet wavelengths (380 to 490 nm) of the FITC filter sets. Fluorescence observations resulting from the use of four combinations of exciter filters are summarized in Table 1. Fluorescence emission of diatoms (Bacillariophyta), green algae (Chlorophyta), yellow-green algae (Chrysophyta), and euglenoids (Euglenophyta) was enhanced by removing the LP-455 filter of the FITC set indicating that peak excitation wavelengths for these algae are below 455 nm.

## Preservative Comparisons

Table 2 summarizes the evaluations of five preservation techniques after a nine-month refrigerated storage period. Gluteraldehyde, as previously suggested by Coulon and Alexander (1972), was the best preservative tested. Cells preserved with "M<sup>3</sup>" generally appeared to have remained in good morphological condition, but some component(s) (probably iodine) of the preservative inhibited fluorescence. Algae preserved with buffered formalin (3% and 5%) appeared to be in relatively poor condition and had obviously undergone substantially more pigment deterioration and morphological deformation than was the case with the other preservatives. Freezing appeared to be a good technique for preserving pigments and for storing samples for future qualitative analyses. The technique proved unsatisfactory for quantitative analyses, however, because of damage to diatom frustules. Almost all of the frustules of *Rhizosolenia* (the dominant diatom taxon in the sample) were broken by the freezing process, although damage to non-diatom algal cells, including several flagellated species, did not appear to be substantial.

Comparative densities of "live" (pigmented) algae in "M<sup>3</sup>" and gluteraldehyde-preserved subsamples with and without the use of fluorescent illumination are shown in Table 3. All counts of "M<sup>3</sup>" and gluteraldehyde-preserved subsamples were similar when conventional illumination was used. Counts of gluteraldehyde-preserved algae with and without the use of fluorescence

illumination were similar except for the blue-green algae. The substantially higher number of blue-green algae observed only with fluorescence illumination was primarily due to small-diameter (about 1  $\mu\text{m}$ ) coccoid cells which rarely were observed in colonies. These organisms could not be differentiated from silt particles with conventional white-light microscopy, but exhibited bright chlorophyll fluorescence. Density estimates of blue-green algae and total phytoplankton were significantly higher (95% confidence) when fluorescence illumination was utilized. The higher number of cryptomonads observed in the "M<sup>3</sup>" preserved subsamples was not statistically significant at the 95% confidence level because of the variance associated with a count of that size (Lund, *et al.*, 1958).

Although gluteraldehyde was the best of the preservation techniques tested, fluorescence in gluteraldehyde-preserved samples was less intense than in live samples. In addition, differences between major groups resulting from their different fluorescence characteristics at different wavelengths was also considerably less distinctive in all preserved samples compared to live samples.

## DISCUSSION

The substantially brighter fluorescence of blue-green algae, red algae, and cryptomonads which occurred at excitation wavelengths between 520 and 560 nm compared to the fluorescence of these algae at excitation wavelengths of 380 to 500 nm was apparently due to light absorption by phycobilin protein molecules (biliproteins). Most types of the biliprotein pigment phycoerythrin

have been shown to have absorption maxima at about 540 nm (O'hEocha, 1962; Braun, *et al.*, 1974). Taxa from all other algal groups examined in this study (and several other studies at our laboratory) fluoresced brightest at excitation wavelengths between 380 and 490 nm which includes the excitation maxima for all known types of algal chlorophyll (Tunzi, *et al.*, 1974).

Fluorescence microscopy offers several taxonomic advantages. The distinct detection of chlorophyll helps one to determine whether a taxon is an alga, a protozoan, or a bacterium. Differentiation of biliprotein-containing algae from other groups is also a valuable taxonomic aid, particularly in samples which contain both coccoid blue-green cells and coccoid green algal cells. Furthermore, the distinction between small unicellular blue-green algal cells and silt particles can in some cases only be adequately made with fluorescence microscopy as was demonstrated by the enumerations comparing preservation techniques.

Fluorescence microscopy offers, in addition to the taxonomic advantages, increased accuracy of algal enumerations by (1) facilitating the detection of algal cells which are obscured by silt and detritus when examined with conventional light and (2) providing an additional criterion for estimating the relative percentages of "live" and "dead" cells in preserved samples. Although a microscopical estimation of the photosynthetic activity of a particular algal cell can only be achieved by autoradiography (Brock, *et al.*, 1975; Paerl, *et al.*, 1976), the distinct presence

of fluorescent chlorophyllous pigments (possibly including chlorophyllides, pheopigments, etc.) is a good indication that a cell was alive at or near the time of collection.

Previous studies by Munro and Brock (1968), and recent studies by Wilde (unpublished results) have indicated that a fluorescent microscope is essential for making an accurate assessment of epipsammic algae (small cells attached to sand grains). This often overlooked assemblage (Round, 1965) appears to be an important component of the total algal community in areas where the euphotic zone coincides with relatively undisturbed sandy sediments. Algae growing epiphytically on liverworts, vascular plants, or macroscopic algae, as well as cells endophytically associated with protozoa can also be clearly observed when fluorescence-inducing illumination is used.

Further refinement of fluorescence microscopical equipment and techniques will undoubtedly result in some additional applications for phycological studies. It might be possible to microscopically assess the physiological condition of algae if functional chlorophyll pigments can be fluorometrically differentiated from chlorophyll degradation products. Food chain studies could also be greatly facilitated by fluorescence microscopy since the technique enhances the observation of pigmented algal cells in the gut and fecal products of grazing invertebrates.

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TABLE 1. Relative Fluorescence of Live Algae at Various Excitation Wavelengths

<i>Filter Set</i>	<i>Excitation Wavelength</i>	<i>Cyano-phyta</i> 7 spp.	<i>Rhodo-phyta</i> 2 spp.	<i>Bacillario-phyta</i> 50 spp.	<i>Chloro-phyta</i> 6 spp.	<i>Crypto-phyta</i> 2 spp.	<i>Eugleno-phyta</i> 2 spp.
1	540-560	***	***	**	*	***	*
2	520-560	***	***	**	*	***	*
3	380-490	*	**	***	***	**	***
4	455-500	*	**	**	**	**	**

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\*\*\* = Very bright

\*\* = medium intensity

\* = dim

TABLE 2. Comparison of Preservation Techniques for  
Use With Fluorescence Microscopy

<i>Technique</i>	<i>Evaluation</i>	
	<i>Preservation</i>	<i>Fluorescence</i>
"M <sup>3</sup> " Preservative	Good	Poor
5% Gluteraldehyde	Good	Good
5% Buffered Formalin	Poor	Fair
3% Buffered Formalin	Poor	Fair
Freezing	Poor	Good

TABLE 3. "Live" Algal Densities (Organisms/ml) of Subsamples Collected in January 1977 and Analyzed in October of 1977

<i>Preservative Illumination</i>	<i>5% "M<sup>3</sup>" White light only</i>	<i>5% Gluteraldehyde White light &amp; fluorescent</i>	<i>5% Gluteraldehyde White light only</i>
Diatoms	1356	1338	1251
Green Algae	349	384	371
Blue-green Algae*	92	1914	78
Cryptomonads	65	39	39
Yellow-green Algae	4	4	4
Dinoflagellates	17	13	13
Total Phytoplankton*	1883	3692	1756

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\* Significant differences between treatments (95% confidence level).

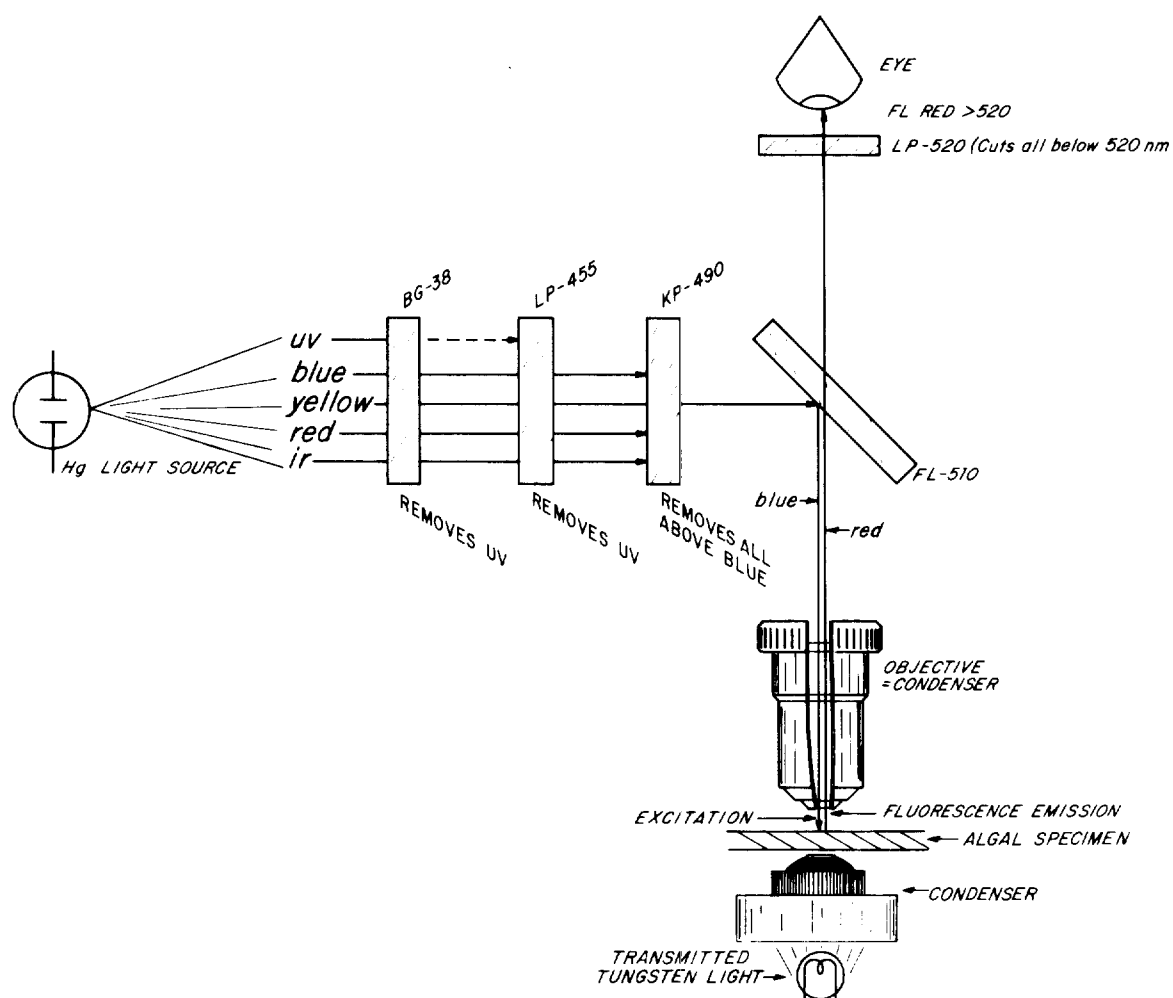


FIGURE 1. Combined Epifluorescence and Transmitted-Light Microscopy